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Provenance of *Ichthyosaura alpestris* (Caudata: Salamandridae) introductions to France and New Zealand assessed by mitochondrial DNA analysis

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The last century has seen an unparalleled movement of species around the planet as a direct result of human activity, which has been a major contributor to the biodiversity crisis. Amphibians represent a particularly vulnerable group, exacerbated by the devastating effects of chytrid fungi. We report the malicious translocation and establishment of the alpine newt (*Ichthyosaura alpestris*) to its virtual antipode in North Island of New Zealand. We use network analysis of mitochondrial DNA haplotypes to identify the original source population as *I. a. apuana* from Tuscany, Italy. Additionally, a population in southern France, presumed to be introduced, is identified as *I. a. alpestris* from western Europe. However, the presence of two differentiated haplotypes suggests a mixed origin. This type of analysis is made possible by the recent availability of a phylogenetic analysis of the species throughout its natural range. We discuss the particulars of both introductions.

Key words: Alpine newt, France, *Ichthyosaura alpestris*, introductions, mtDNA, New Zealand

INTRODUCTION

Humans have been responsible for translocating plants and animals since prehistoric times (King, 1985; Vitousek et al., 1997; McDowall, 2011) and with modern transport the number of translocations has increased thereby adding to the global biodiversity crisis (Burdick, 2006; Elton, 2000; Parkes & Murphy, 2003). With the colonial era came protracted, deliberate, long-distance introductions, for which we continue to pay the price today (Pysek & Richardson, 2010). Colonisation of the New World and Australasia was accompanied by activities of "acclimatisation societies", whose mission was to re-create a little Europe on the other side(s) of the world (McDowall, 1994; Allen & Lee, 2006). Although a conservation ethic replaced this desire during the 20th century, introductions continue in the name of (often misguided) biological control (Easteal, 1981; Kats & Ferrer, 2003), and as an indirect result of the pet trade (Brede et al., 2000; Fontelles et al., 2011; Meilink et al., 2015) or scientific research (Arntzen & Thorpe, 1999; Rebelo et al., 2010; Measey et al., 2012). Herpetological animal enthusiasts have played their part in this practice (Kuzmin, 1994; Kraus, 2009). This paper concerns two introductions of a European amphibian species, the alpine newt, *Ichthyosaura alpestris* (Laurenti, 1768), to France and to New Zealand, and their identification by molecular genetic means.

The natural distribution of the alpine newt includes a large part of Europe: from northwestern France to western Russia, and southern Denmark to southern Italy and Greece (Sillero et al., 2014). There is a relictual distribution along the northern edge of Spain [subspecies *I. a. cyreni* (Wolterstorff, 1932)]. Post-glacial expansion northwards into the British Isles was presumably prevented by the English Channel. A combination of preference for cooler conditions and competitive exclusion may have left most of the Iberian peninsula and southwestern France without alpine newts (Denoël, 1996, 2005).

Many introduced populations of alpine newts have been reported in different parts of Europe (Sillero et al., 2014). The UK has several naturalised populations, the oldest dating back to the 1920s (Bell & Bell, 1995; Lever, 2003). Many of these probably involved subsequent translocations within the UK, but some new introductions from the continent also occurred (Bell & Bell, 1995). The wide distribution of *I. alpestris* in the central parts of the

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Netherlands is attributed to introductions (van Delft, 2009).

An isolated population of alpine newts found in the Peñalara Natural Park in the Guadarrama mountains (north of Madrid) has been considered autochthonous on the basis of an early record for central Spain ('Madrid', Mertens & Müller, 1928). Since that time, however, the species was not reported in the Madrid province again until July 1984, when Lope & Cuadrado (1985) observed alpine newts at Peñalara at low abundance in a restricted area (see also García-París et al., 1989). A decade later the species had spread over a wide area and occurred in more than 20 ponds, at a density of up to nine adults per hectare (Martínez-Solano et al., 2006). Population density is still on the increase and alpine newts are colonising adjacent sectors of the park (Martínez-Solano et al., 2003). The spatial-temporal signature of the expansion of the Peñalara population is more consistent with a recent introduction of a small number of individuals than autochthonous status, so we suggest that the presence at Peñalara is unrelated to the early record in Mertens & Müller (1928). Consistent with this view, the Peñalara alpine newt population had the lowest values of genetic diversity in the allozyme study of Arano et al. (1991) and only a single mtDNA haplotype in the three individuals analysed by Recuero et al. (2014). Small genetic distances to populations in Asturias, northern Spain, suggest that it concerns a local translocation as alluded to earlier (Arano et al., 1991; Lever, 2003).

In southern France, an introduction of alpine newts of unknown origin occurred in a locality near Le Cros, in the Larzac limestone plateau (department Hérault) between the 1970s and the 1990s (Denoël, 2005; Geniez & Cheylan, 2012) (Fig. 1). Some individuals dispersed to nearby ponds and established a breeding population in one of them (Denoël, 2005). More dramatic is a recent report of a deliberate smuggling into New Zealand, where a population has been flourishing locally (near Waihi, Waikato, North Island) for 10–15 years (Fig. 1). Eradication efforts are underway using netting, box-traps, drift-fencing, pitfall traps, sniffer dogs and pond-emptying. More than 3000 newts have already been removed over a two-year operational period (Ministry of Primary Industries, New Zealand).

The provenance of translocated fauna can effectively be addressed using DNA markers when the distribution of genetic variation in native populations is well established. A recent survey of *I. alpestris* from throughout its range, including the main subspecies, provides this background (Recuero et al., 2014) along with more extensive but less detailed information (Sotiropoulos, 2007a,b; Lužnik et al., 2011). Here, we use mtDNA sequences from individuals of introduced populations in France and New Zealand to identify their potential origins.

MATERIALS AND METHODS

Adult alpine newts were caught by dip-netting in ponds. Tail tips were taken from four individuals from Bagnelades pond in Larzac, France (43°51'N, 3°21'E; 735 m a.s.l., municipality of Le Cros) in April 2014 and from four individuals from near Waihi, Waikato, New Zealand (specific site information withheld at the request of the Ministry of Primary Industries, New Zealand) in September 2013. DNA was isolated using a standard Chelex (BioRad) protocol (Casquet et al., 2012). Two mitochondrial genes (as in Recuero et al., 2014) were amplified: 596 bp of 16S using primers 16Sar and 16Sbr (Simon et al., 1994) and 957 bp of ND4, tRNA-His, tRNA-Ser and tRNA-Leu using primers ND4 and Leu (Arévalo et al., 1994). PCR reactions contained 0.5 µM each primer and 1 x MyFi Mix (Bioline) in a total volume of 10 µl and were cycled in an Eppendorf Mastercycler ProS: 94°C for 180 s followed by 35 cycles of 94°C for 30 s, 50°C (16S) or 56°C (ND4) for 30 s, 72°C for 60 s, with a final extension of 72°C for 240 s. Amplified DNA was purified using a MEGA quick-spin total fragment DNA purification kit (iNtRON), quantified using a Nanodrop ND-1000 spectrophotometer and sequenced on an ABI 3730xl DNA Analyser (Genetic Analysis Service, Department of Anatomy, University of Otago) using one or both PCR primers. The new sequences from eight individuals have been submitted to GenBank (accession numbers KR107542–KR107557). Sequences were aligned by hand relative to an existing database of 136 sequences, including seven sequences from the outgroup species *Lissotriton boscai* (Lataste, 1879), *L. italicus* (Peracca, 1898) and *Ommatotriton vittatus* (Gray, 1835) (Recuero et al., 2014). This dataset was subjected

Fig. 1. Introduced alpine newts: *Ichthyosaura alpestris apuana* from near Waihi, Waikato, New Zealand (left panel, photo: J. Reardon) and *I. a. alpestris* from Larzac, southern France (right panel, photo: M. Denoël). Both pictures show a male during the breeding season.

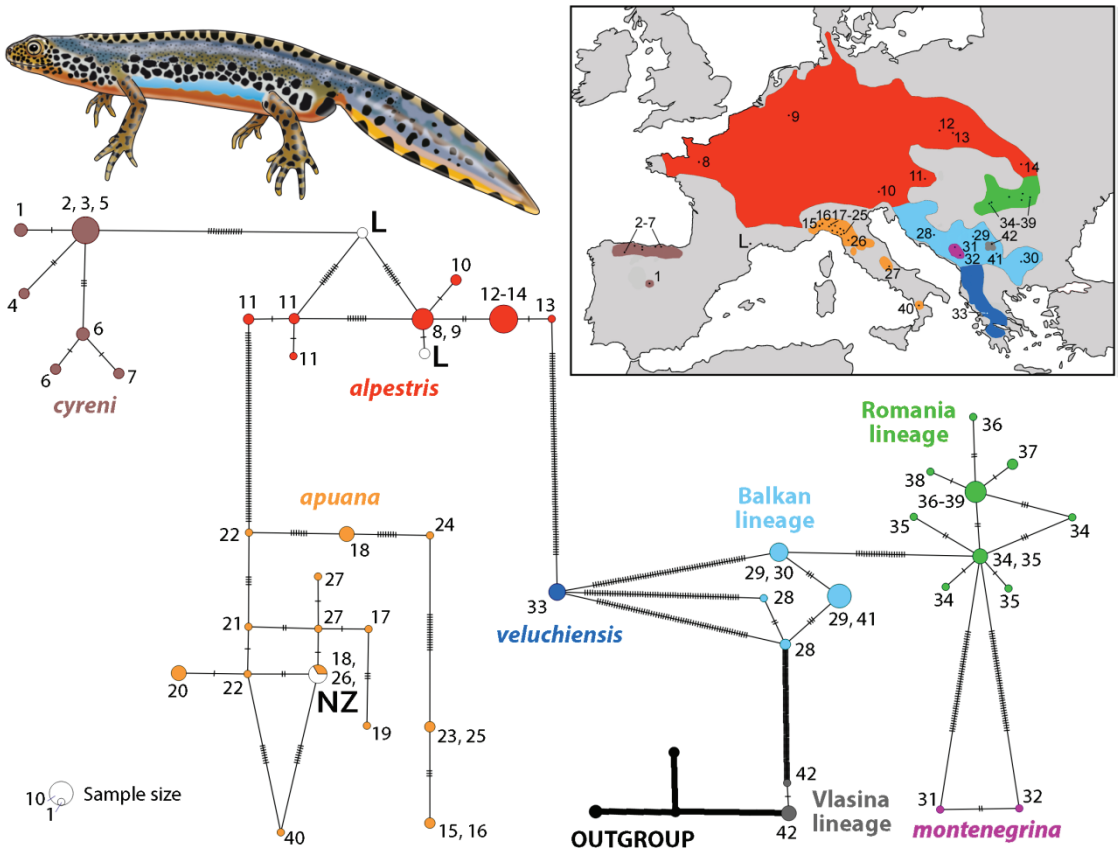

Fig. 2. Minimum spanning network of mitochondrial DNA sequences of *Ichthyosaura alpestris* (ND4 and 16S). Bars represent nucleotide substitutions. The colour scheme follows that of Recuero et al. (2014) with introduced populations shown in white. Mitochondrial DNA sequences from introduced populations in France (L) and New Zealand (NZ) affiliate with *I. a. alpestris* and with *I. a. apuana*, respectively. Numbers refer to the localities shown on the mtDNA haplotype distribution map (insert, after Recuero et al., 2014, from which also the drawing is taken).

to a phylogenetic network analysis (minimum spanning network) with PopART (Leigh & Bryant, 2015; software available at: http://popart.otago.ac.nz) under default settings, with all sequences incorporated.

RESULTS

Eight new sequences of common length 1442 bp were obtained from non-native populations of alpine newts in France and New Zealand. The haplotype network groups individuals from Larzac, France with *I. a. alpestris* and those from New Zealand with *I. a. apuana* (Bonaparte, 1839) (Fig. 2). The French material shows two distinct haplotypes: one differs by a single nucleotide site from a haplotype found in localities 8–9 (France and Germany) in Recuero et al. (2014) and the other does not closely match any naturally occurring population that has been sampled. This haplotype is 10 mutational steps from the closest *I. a. alpestris* haplotype and 14 from the most similar *I. a. cyreni* haplotype (Fig. 2). New Zealand haplotypes are identical to some from Tuscany, Italy (localities Cardoso at 44°01' N, 10°29' E and Seravezza at 44°00' N, 10°14' E) (Fig. 2). A wider sampling with 16S sequence data confirms the position of the Larzac individuals in the '*alpestris* plus *cyreni*' clade (Fig. 3A). In the minimum spanning network they group with the most common *alpestris* haplotype (*n*=2) and with a haplotype from northeast Italy and Slovenia (*n*=2) (Fig. 3B).

DISCUSSION

The new mtDNA sequences indicate that the Larzac French population (at least in part) represents the nominotypical subspecies whereas the New Zealand introduction is incontrovertibly *I. a. apuana*. The nominotypical subspecies has a range from France to Ukraine, with little genetic differentiation (Recuero et al., 2014), which makes precise inference of the population of origin difficult. Still, the Larzac individuals cluster most closely with the western representatives (populations 8–11 from France, Germany, Austria and Hungary in Recuero et al., 2014, Fig. 2), which makes provenance from e.g., Poland, Romania or the Balkans less likely. The two haplotypes from Larzac are both new, which suggests that the introduction has been from part of the *I. a. alpestris* range that is as yet not sampled. The western Alps are mentioned as a possible source (J. Gabrion, pers. comm.).

It is noteworthy that Larzac possesses two distinct haplotypes, a pattern that is not found anywhere else. Moreover, one divergent haplotype (Fig. 2) is genetically almost equidistant between *cyreni* and *alpestris*. At first sight, this may have suggested an introduction on top of

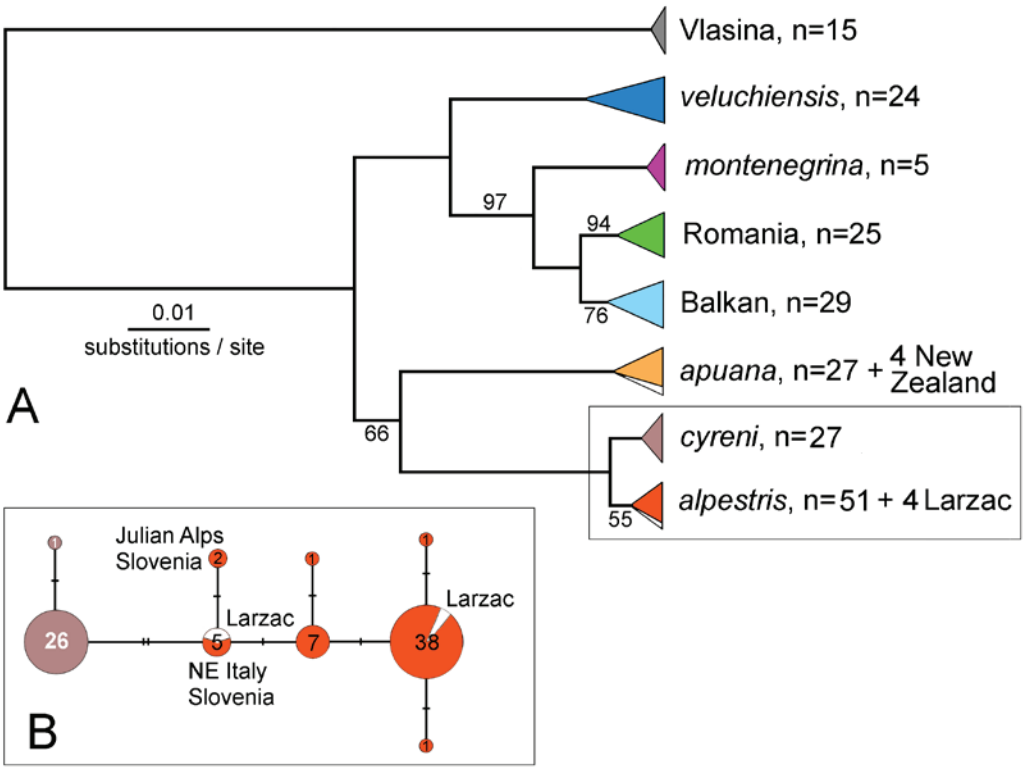

Fig. 3. A) Phylogenetic analysis for 16S sequence data. Values on branches are Bayesian posterior probabilities (expressed as %) and are shown only if not 100. B) Minimum spanning network for 16S sequences in the '*alpestris - cyreni*' clade. For details see Appendix 1.

an unknown relictual population in between the *alpestris* and *cyreni* ranges, i.e. at Larzac. Although this seems an unlikely scenario, a similar introduction has happened before with *Triturus cristatus* (Laurenti, 1768) in the northeast of the French department Mayenne (Arntzen et al., 2010). However, we consider an old presence of the alpine newt at Larzac very unlikely because the Larzac pond (Bagnelades) was sampled in the early 1970s by J. Gabrion and only the palmate newt *Lissotriton helveticus* was recorded there (Gabrion et al., 1977). The first mention of the alpine newt in Bagnelades was by V. Fradet & R. Duguet in 1995 (in: Geniez & Cheylan, 2012). The atlas of amphibians of Languedoc-Roussillon presents no other historical data on the alpine newts of Larzac and surrounding areas and considers it an introduction (Geniez & Cheylan, 2012). All ponds of the Hérault section of Larzac have been intensively searched for amphibians (Geniez & Cheylan, 2012; Denoël & Ficetola, 2014, 2015) and the alpine newt was only abundant in Bagnelades pond whereas only isolated or few individuals were recorded in three nearby ponds (Denoël 2005; M. Denoël. pers. obs.). We consider it not impossible that several introductions were made in Bagnelades. Two independent sources informed us that alpine newts were bred in captivity and then released at several occasions in Larzac by a local researcher but without confirmation of the release site(s) (J. Gabrion & G. Hanula, pers. comm.). These alpine newts came at least in part from the Alps and were studied in a research laboratory from the 1960s to the seventies or early eighties (J. Gabrion, pers. comm; see also e.g., Sentein, 1966, 1970). It is possible that several populations were used for the laboratory experiments, then explaining the presence of two haplotypes in Bagnelades. The origin of the Larzac population thus remains uncertain until further data from western Europe, particularly the Alps, become available. Using more variable markers may also help to assign the population(s) of origin.

The New Zealand population may result from a one-off introduction directly from Europe. The genetic data provide a full match with populations from Tuscany, Italy. While the New Zealand alpine newts mostly lack the gular black spots that are typical for subspecies *apuana,* populations without this character state have been found across the range, including Tuscany (Ferracin et al., 1980). *Ichthyosaura a. apuana* is particularly colourful and hence possibly more frequently traded than other subspecies. However, alpine newts from Calabria, southern Italy, mentioned as a source of trade by the IUCN (Arntzen et al., 2009), can be excluded as the origin of the New Zealand population (Fig. 2, locality 40) on account of the several substitutions between the Calabria and New Zealand haplotypes.

In Europe, many of the introduced *I. alpestris* populations are by now well established. Population sizes may be substantial and newts have dispersed to nearby ponds, rendering the species' presence virtually irreversible (Bell & Bell, 1995; Bosch & Martínez-Solano, 2003; Denoël, 2005; Martínez-Solano et al., 2003; van Delft, 2009). In New Zealand, the introduced newts may pose a threat to endemic and endangered leiopelmatid frogs (Newman et al. 2010), either directly by predation and competition, or indirectly through the vectoring of disease. Of particular concern is Archey's frog, *Leiopelma*

archeyi, Turbott, 1942, whose main stronghold is the Coromandel Peninsula, adjacent to the area with *I. alpestris* (Bell, 2010). We think that the effect of competition and predation will be minor because Archey's frogs are terrestrial breeders, inhabiting the cooler moist native forest habitat at 100–1000 m. a.s.l., whereas *I. alpestris* usually breeds in ponds and only rarely in running water (Breuil & Parent, 1987). However, *I. alpestris* uses similar (deciduous) woodland in northeastern France (G.P. Wallis, pers. obs.) and elsewhere (Denoël & Ficetola, 2008), where it can potentially use permanent small seeps and pools. The larger, more widespread and more aquatic *L. hochstetteri* Fitzinger, 1861 is also found on the Coromandel, usually close to streams (G.P. Wallis, pers. obs.). The terrestrial habitat of the species has the potential to be overlapping in this forested area, but as yet alpine newts appear to be restricted to the lowland pastoral site of introduction.

New or unknown parasites and pathogens possibly introduced along with the newts may pose more of a threat. *Ichthyosaura alpestris* from the Cantabrian mountains has been recently reported to be experiencing high mortalities and population declines after infection with a ranavirus (Price et al., 2014). The chytrid fungus *Batrachochytrium dendrobatidis* Longcore, Pessier & Nichols, 1999 is one of the major threats to amphibian populations worldwide (Fisher et al., 2011) and is reported to have infected *I. alpestris* (Spitzen-van der Sluijs et al., 2014). Moreover, *I. alpestris* has been identified as an asymptomatic vector in the spread of this disease in introduced populations in the United Kingdom (Arntzen et al., 2009). Initial screens by PCR for chytrid in the newt introduced to New Zealand show >70% to be infected (J. Laycock, pers. comm.).

Chytrid fungus has been described in New Zealand, first in naturalised Australian *Litoria raniformis* Keferstein, 1867 (Waldman et al., 2001) and later in native *Leiopelma* sp. (Shaw et al., 2013). While leiopelmatid frogs have low susceptibility (Ohmer et al., 2013), and *L. hochstetteri* may even show resistance (Moreno et al., 2011) to *Batrachochytrium dendrobatidis*, there are many different strains that could have varied effects (Herbert et al., 2011). The discovery of yet another new chytrid specifically affecting salamanders (*Batrachochytrium salamandrivorans* Martel, Blooi, Bossuyt & Pasmans, in Martel et al., 2013) shows that caution should be taken. Yet elsewhere, *I. alpestris* individuals have been recorded as dying rapidly after contamination with this new pathogen (Martel et al., 2014). Intriguingly, *L. archeyi* went into marked decline in the late 1990s (Bell, 2010), coinciding with some frogs testing positive for chytrid, and possibly coinciding with the nearby introduction of *I. alpestris*.

Within Europe it is not always clear whether a population is introduced or perhaps relictual. One way to assess the likelihood of an introduction *per se* is to measure effective population size, as was done with *Triturus cristatus* in France (Arntzen et al., 2010). In this case, genetic variation of the population of interest was high, rendering an introduction as an unlikely explanation. Conversely, there can be no doubt that the New Zealand *I. alpestris* population is introduced, as with *Lissotriton vulgaris* recently discovered in Australia (Tingley et al., 2015). The naturalisation of newt species in both hitherto caudate-free Australasian continents exemplifies the global reach of anthropogenic translocation. In New Zealand, the alpine newt has officially been declared an unwanted organism (http://www.biosecurity.govt.nz/pests/alpinenewt). Invasive populations often undergo a lag phase before exponential population growth takes place (Aagaard & Lockwood, 2014; Crooks & Soulé, 1999). As a result, the cost of management of invasive species increases exponentially over time, while the potential for successful eradication decreases (Pitt et al., 2005). This emphasizes the need to fund eradication schemes at an early stage of invasion and prevent secondary human-aided spread.

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APPENDIX

Appendix 1.

Phylogenetic analysis of 16S sequence data. The full 16S alignment (*n*=211 sequences, no outgroups) with sequences from the present study as well as those available from GenBank (see Sotiropoulos et al., 2007ab; Lužnik et al., 2011) was reduced to one representative of each unique haplotype (n=91 sequences) with the online tool FaBox (Villesen, 2007). Then, we reconstructed a gene tree with the software BEAST v.1.8.1 (Drummond et al., 2012). We used jModeltest v.2.1.1 (Darriba et al., 2012) to find the optimal nucleotide substitution model for this reduced dataset (TPM2uf+I+G), which was approximated as GTR+I+G in BEAST. We used a Bayesian Skyline Plot as a coalescent prior with a strict molecular clock and ran the analysis for 50,000,000 generations, sampling genealogies and parameters every 5,000th generation, resulting in 10,000 trees. Convergence was assessed by inspection of the log file in Tracer (Rambaut et al., 2014), and a maximum clade credibility consensus tree was subsequently computed with TreeAnnotator v.1.8.1 (distributed as part of the BEAST package) after removal of the first 10% of the genealogies as burn-in. The 82 sequences representing the '*alpestris* plus *cyreni*' clade were subjected to a network analysis with PopART as above (Leigh & Bryant, 2015; details see text).